Complement Activation Correlates With Disease Severity and Contributes to Cytokine Responses in Plasmodium falciparum Malaria

Complement activation correlates to disease severity and contribute to cytokine response in *falciparum* malaria

Aase Berg[1,2,3§], Kari Otterdal[4§], Sam Patel[2], Miguel Gonca[2], Catarina David[2], Ingvild Dalen[5], Stig Nymo[6,7,8], Margareta Nilsson[6], Thor Ueland[4,9,10,11], Mauro Prato[12,13], Giuliana Giribaldi[14], Tom Eirik Mollnes[6,7,8,9,10], Pål Aukrust[4,,9,10,15], Nina Langeland[3,16], Per H. Nilsson[6].

[1]Department of Medicine, Stavanger University Hospital, 4011 Stavanger, Norway

[2]Department of Medicine, The Central Hospital of Maputo, Maputo, Mozambique

[3]Department of Clinical Science, University of Bergen, 5020 Bergen, Norway

[4]Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, 0372 Oslo, Norway

[5]Department of Research, Stavanger University Hospital, 4011 Stavanger, Norway

[6]Department of Immunology, Oslo University Hospital Rikshospitalet, 0372 Oslo, Norway

[7]Research Laboratory Nordland Hospital, 8092 Bodø, Norway

[8]Faculty of Health Sciences, University of Tromsø, 9019 Tromsø, Norway

[9]K.G. Jebsen Inflammatory Research Center, University of Oslo, 0424 Oslo, Norway

[10]Faculty of Medicine, University of Oslo, 0316 Oslo, Norway

[11]K.G. Jebsen Thrombosis Research and Expertise Center, University of Tromsø, 9019 Tromsø, Norway

[12]Department of Neuroscience, University of Torino, Turin, Italy

[13]Department of Public Health and Pediatric Sciences, University of Torino, Turin, Italy

[14]Department of Oncology, University of Torino, Turin, Italy

[15]Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital Rikshospitalet, 0372 Oslo, Norway

[16]Department of Medicine, Haukeland University Hospital, 5021 Bergen, Norway

Correspondence to: Per H. Nilsson, Department of Immunology, Oslo University Hospital Rikshospitalet, 0372 Oslo, Norway, per.nilsson@rr-research.no

§ AB and KO contributed equally to the manuscript.

E-mail addresses:

AB: beaa@sus.no

KO: kari.otterdal@rr-research.no

SP: sampatelmz@gmail.com

MG: miguel_gonca@hotmail.com

CD: catarinadavid@yahoo.com

ID: ingvild.dalen@sus.no

PN: per.nilsson@rr-research.no

TU: thor.ueland@medisin.uio.no

MP: mauro.prato@unito.it

GG: giuliana.giribaldi@unito.it

TEM: t.e.mollnes@medisin.uio.no

PA: pal.aukrust@rikshospitalet.no

NL: nina.langeland@mofa.uib.no

SN: stig.nymo@gmail.com

MN: margareta.nilsson@rr-research.no

Abstract

Background. *Plasmodium (P) falciparum* malaria is a major life-threatening infectious disease associated with systemic inflammation. Heme and its detoxification product hemozoin are inflammatory stimuli during the intraerythrocytic phase of the *P. falciparum* life cycle. The aim of this study was to investigate the impact of complement activation and its possible relation to cytokine responses in malaria pathology.

Methods. Blood samples from 131 patients with confirmed *P. falciparum* malaria, and plasma from human whole blood incubated *ex vivo* with hemin (heme oxidized form) or natural hemozoin were analyzed for complement activation, measured as C3bc, C5a, and the soluble terminal complement complex C5b-9 (sC5b-9) through ELISA, and for cytokines using a multiplex assay. Complement dependency on cytokine release was studied using specific inhibitors of C3, C5 and the C5a receptor 1 (C5aR1).

Findings. sC5b-9 was significantly increased in the malaria cohort as compared to healthy controls. The sC5-9 levels positively correlated to disease severity ($p<0.05$), and with levels of certain cytokines, and in particular IL-8/CXCL8 ($r_s=0.52$, $p<0.01$), that were significantly elevated in these patients. Hemin and hemozoin induced substantial complement activation and release of cytokines, in particular IL-8/CXCL8, *ex vivo*. All complement inhibitors significantly blocked the IL-8/CXCL8 increase, with similar effects of C3, C5 and C5aR1 inhibition, showing that these effects are mediated through C5aR1 activation.

Interpretations. *P. falciparum* malaria was associated with a systemic complement activation correlated with levels of certain inflammatory cytokines *in vivo*, of which IL-8/CXCL8 was particularly prominent. Hemin and hemozoin were potent complement activators *ex vivo*,

inducing a robust IL-8/CXCL8 response that was complement-dependent and mediated through C5aR1.

Funding.

This study was financially supported by The Research Council of Norway, The Norwegian Council on Cardiovascular Disease, The Western Norway Regional Health Authority, The Northern Norway Regional Health Authority, The Southern and Eastern Norway Regional Health Authority, The Odd Fellow Foundation, The University of Turin (intramural funding) and the European Community's Seventh Framework Programme under grant agreement n°602699 (DIREKT).

Introduction

Plasmodium falciparum (*P. falciparum*) malaria is a major cause of mortality in sub-Saharan Africa. About 750,000 to 1.13 million people die globally every year [1, 2]. In light of those challenges, together with increasing drug and insecticide resistance, studies of the host's immunological and inflammatory responses during *falciparum* malaria are important. While a balanced immune response could be beneficial for the host, leading to increased clearance of the parasite, overwhelming inflammatory responses could lead to tissue damage and deleterious effects for the infected patient. The characterization and regulation of this delicate balance between beneficial and detrimental immune responses during *falciparum* malaria is at present not clear.

The complement system is part of the innate immune defense mechanisms against microbes and consists of more than 50 membrane-bound and soluble proteins [3, 4]. Recent studies suggest that complement can be regarded as a surveillance system that quickly can be activated by sensing of danger to the host, by both sterile and non-sterile stimuli, and thereby contribute to maintain tissue homeostasis and promote tissue repair [5]. On the other hand, undesired or uncontrolled complement activation can induce tissue damage and organ dysfunction in the host, as sometimes seen in septicemia and various autoimmune disorders [6, 7]. Studies in both humans and animal models have shown extensive complement activation during malaria infection [8, 9, 10, 11] though data of terminal complement cascade activation, with generation of the terminal complement complex (TCC), are scarce. In addition, it is still not clear how malaria parasites induce complement activation and how complement activation can activate downstream inflammatory pathways during malaria infection.

An important stimulus in malaria immunopathology is crystalline hemozoin, which is produced as a heme detoxification product after hemoglobin catabolism during the intra-erythrocytic phase of *Plasmodium* life cycle and released together with the parasite and hemoglobin upon rupture of infected erythrocytes [12, 13]. Ferric heme (hemin) is liberated from hemoglobin due to oxidation of the heme-moiety within hemoglobin [14, 15]. Hemozoin crystals and monomeric hemin have been proposed as modulators of inflammation in malaria pathology and have been shown to induce leukocyte activation with generation of inflammatory cytokines [13, 16]. Under certain conditions, the release of several cytokines, in particular interleukin (IL)-8/CXCL8, is directly dependent on complement activation [17]. However, it remains unclear so far whether hemin and hemozoin directly induce complement activation and whether complement activation is crucial in cytokine response following hemozoin/hemin release.

The aim of this study was to elucidate the role of complement activation during *P. falciparum* infection. Plasma complement activation products were measured and analyzed for correlation to disease severity and cytokine plasma levels in *P. falciparum*-infected patients admitted to the Central Hospital of Maputo in Mozambique. Furthermore, the interaction between malaria and complement was studied *ex vivo* by incubating hemin and hemozoin in non-immune human whole blood using a model designed for investigation of crosstalk between complement and the remaining inflammatory network [18]. By employing complement inhibitors, we were able to document a causal relation between hemin- and hemozoin-mediated complement activation and the subsequent release of cytokines, in particular IL-8/CXCL8.

Methods

Study design and participants

The study population has previously been described [19]. Briefly, during two malaria seasons, from 8th January 2011 to 31st March 2011 and from 7th November 2011 to 14th March 2012 we collected clinical data and blood samples from all adult nonpregnant patients admitted to the Central Hospital of Maputo, Mozambique with an axillary temperature ≥ 38°C and/or suspected malaria. Of the 212 screened patients, 131 were found to have *P. falciparum* malaria as assessed by PCR (n=129) or antigen test/slides (n=2). Of the 131 malaria patients, 70 were found to be HIV-infected as assessed by PCR and/or serological tests [19]. Severe malaria and very severe malaria were defined as more than one and three or more malaria severity criteria, respectively, modified from the definition of severe malaria by WHO [19, 20]. For comparison, health care workers and their family and friends were included as controls (n=52), provided that they were well-being and had a healthy appearance as evaluated by the researcher. All included controls were negative for *P. falciparum* and HIV infection.

Collection of blood samples and preparation of plasma from patients and controls

Blood samples from patients and healthy controls were collected from a pre-alcohol-cleaned peripheral vein into pyrogenic-free tubes with EDTA. The EDTA vacutainer tubes were turned gently, placed immediately on melting ice, and centrifuged within 30 minutes at 2000*g* for 20 minutes to obtain platelet-poor plasma. Plasma was aliquoted and stored first at -20°C for 24 hours; then at -80°C. The samples were thawed only once and were analyzed for complement and cytokines as described below.

Whole blood sampling for *ex vivo* experiments

Whole blood was obtained from nonimmune healthy adult volunteers who had received no medication for at least 10 days. Blood was drawn from an antecubital vein into 4.5 ml sterile polypropylene cryotubes (Nalgene NUNC, Roskilde, Denmark) containing the thrombin specific inhibitor lepirudin (Refludan; Pharmion ApS, Copenhagen, Denmark) at a final concentration of 50 μg/ml. Lepirudin was used as anticoagulant in all *ex vivo* experiments. The samples were analyzed for complement and cytokines as described below.

Complement assays

The complement activation products C3bc and the sC5b-9 were analyzed using enzyme-linked immunosorbent assays (ELISAs) as described in detail previously [21]. Briefly, the assays were based on monoclonal antibodies detecting neo-epitopes exposed after activation of the components, thus specifically measuring only the activated component. The amount of activation products present is related to an international standard defined to contain 1000 complement arbitrary units (CAU)/ml. C5a was analyzed in plasma using the commercial ELISA DuoSet kit (R&D systems, Minneapolis, MN) according to the manufacturer protocol. Detected C5a is expressed as ng/ml.

Cytokine assays

Plasma samples from whole blood *ex vivo* cultured (see below) were analyzed using a 27-Plex Panel multiplex cytokine assay (Bio-Rad Laboratories Inc., Hercules, CA) as previously described [22]. In the present study the following cytokines were analyzed for their correlation to plasma sC5b-9: IL-8/CXCL8, monocyte chemotactic protein 1 (MCP-1)/CCL2, macrophage inflammatory protein (MIP)-1β/CCL4, and IL-6.

Preparation of hemin and hemozoin

Hemin (ferriprotoporphyrin IX; Sigma-Aldrich, St. Louis, MO), the ferric oxidized form of heme, was dissolved in 20 mM NaOH to 10 mg/ml and kept in dark at 4°C until use. Hemozoin was prepared from *P. falciparum* parasites (Palo Alto strain; mycoplasma free), which were kept in culture as previously described [23]. After centrifugation at 5000*g* on a discontinuous Percoll-mannitol density gradient, natural hemozoin was collected from the 0–40 % interphase and washed and centrifuged at 5000*g* five times with 10 mM KH_2PO_4 (pH 8.0) containing 10 mM mannitol at 4°C, and once with PBS. After quantification of heme content by luminescence [24], natural hemozoin was stored at 20 % (v/v) in PBS at -20°C.

Complement inhibitors

For complement inhibition, compstatin (Cp40 analogue (*d*Tyr-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-His-Arg-Cys]-mIle-NH2) was used to specifically target C3 [25]. C5 was blocked using eculizumab (Alexion Pharmaceuticals, Inc. Cheshire, CT) and the C5a-receptor 1 (C5aR1, CD88) inhibitor AcF[OPdChaWR], synthesized as previously described [26], was used for blockade of the C5aR1. Compstatin and C5aR1 inhibitor were kindly provided by prof. John Lambris, University of Pennsylvania.

Ex vivo whole blood activation

The inflammatory response in whole blood was studied by incubating hemin or hemozoin in 1.8 ml round-bottom sterile polypropylene NUNC cryotubes (Nalgene NUNC) on rotation for 4 hours at 37°C. Following incubation, EDTA was added to a final concentration of 20 mM and the blood was centrifuged to platelet-poor plasma (3000*g* for 20 minutes at 4°C) which was immediately isolated and stored at -80°C until further analysis.

Complement-dependent cytokine response to hemin and hemozoin

To characterize the hemin-induced cytokine production in blood and to study the complement dependency thereof, hemin 1000 μg/ml and hemozoin 10 μg/ml were incubated in lepuridin-anticoagulated whole blood supplemented with the complement inhibitors compstatin (20 μM), eculizumab (100 μg/ml), and C5aR1 inhibitor (20 μM). The inhibitors were pre-incubated in whole blood for 5 minutes prior to the addition of hemin or hemozoin. Incubation was carried out on rotation at 37°C, four hours for complement activation and cytokine generation and 15 minutes for expression of CD11b. Following incubation, EDTA was added and plasma was isolated as described above. Zymosan A 1000 μg/ml (from *Saccharomyces cerevisiae,* Sigma-Aldrich) and ultrapure LPS 100 ng/ml (from *E. coli* strain O111:B4; InvivoGen, San Diego, CA) served as positive controls for complement activation and cytokine generation, respectively.

Expression of CD11b

Expression of CD11b on granulocytes and monocytes was detected using flow cytometry. Whole blood was pre-incubated with complement inhibitors as described above and incubated with hemin 1000 μg/ml for 15 minutes. Cells were then fixed using 0.5 % (v/v) paraformaldehyde for 4 minutes at 37°C, before staining with anti-CD11b-PE (D12, BD Biosciences), anti-CD14-FITC (MφP9, BD Biosciences) and anti-CD45-PerCP (2D1, BD Biosciences) for 15 minutes at room temperature. Red blood cells were then lysed and the samples washed and assayed on a FACSCalibur flow cytometer (BD Biosciences). Leukocytes were gated as $CD45^+$ cells, and granulocytes and monocytes were separated based on their CD14 expression. Data were collected using Cell Quest Pro (v 5.2.1, BD Biosciences) and analyzed with FlowJo (version 10, Tree Star).

Statistical analysis

Differences between the patient groups were tested by chi square test for categorical variables and with Mann-Whitney test for continuous data. Correlations between sC5b-9 and cytokines were investigated using Spearman's Rank Order Correlation. Results from the *ex vivo* experiments are presented in scattered plots as means and 95 % confidence intervals. For statistical analysis, the values from hemin and hemozoin were compared to those of buffer controls and significance was calculated using paired *t*-test. If significantly separated ($p< 0.05$), hemin and hemozoin were further compared to hemin and hemozoin supplemented with complement inhibitors using one-way ANOVA followed by Dunnett´s multiple comparisons test using GraphPad Prism version 6.0d for MAC OS X (GraphPad Software, La Jolla CA). When pairing was significantly effective ($p<0.05$), statistical significance was calculated using repeated measurements ANOVA with Dunnett´s post-hoc test and corrected for unequal sphericity (Geisser-Greenhouse correction).

Role of the funding source

The sponsors had no role in study design, data collection, data analysis, data interpretation, or writing the report. The corresponding author had full access to all data in the study and the final responsibility for the decision to submit for publication.

Ethical considerations

The study was designed and performed according to the Helsinki Declaration from the 59th WMA General Assembly, Seoul, Republic of Korea, October 2008. The National Ethical Committee at the Ministry of Health in Mozambique and the Regional Ethical Committee in Eastern Norway approved the study. A signed consent or fingerprint was obtained from patients or their next of kin and from the healthy controls. For the *ex vivo* study in whole

blood, informed written consent was obtained from each donor, and the study was approved by the Regional Ethical Committee of South-Eastern Norway Regional Health Authority.

Results

Demographic patient data

The patient population has previously been described [19]. Briefly, a total of 212 non-pregnant adults with fever and/or suspected malaria were screened and 131 had *P. falciparum* malaria, and of these, 70 were co-infected with HIV, and two were co-infected with *P. vivax* and *P. malariae,* respectively. In addition, 52 healthy controls were included (see Methods).

In vivo complement activation during *P. falciparum* infection

Patients with *P. falciparum* malaria had markedly raised plasma levels of sC5b-9 as compared with healthy controls with increasing levels according to disease severity (Figure 1). In fact, patients with the most severe malaria had significantly raised levels of sC5b-9 not only as compared with healthy controls ($p<0.001$), but also as compared with malaria patients with less severe disease ($p<0.05$) (Figure 1). Seventy of the patients were co-infected with HIV-1, and we have previously shown that these patients were characterized by more severe malaria disease [19]. HIV-co-infected patients had moderately higher sC5b-9 levels than the other malaria patients (2.0 CAU/mL versus 1.4 CAU/mL, data are given as medians and 25^{th}-75^{th} percentiles, $p<0.01$).

Correlation between complement activation and cytokines

We have previously published data on cytokine levels in these malaria patients [22]. Plasma levels of sC5b-9 significantly correlated to the levels of four of the cytokines that were raised in malaria patients as compared with controls: IL-6 ($r_s=0.63$), IL-8/CXCL8 ($r_s=0.52$), MCP-1/CCL2 ($r_s=0.62$) and MIP-1β/CCL4 ($r_s=0.62$), $p<0.01$ for all comparisons (Figure 2). IL-1

receptor antagonist, IL-9, IL-10, eotaxin/CCL11, interferon inducing protein-10 (IP-10)/CXCL10 were also increased in the patients [22], but did not correlate with sC5b-9.

Hemin-induced inflammatory response in human whole blood *ex vivo*

Complement activation

Hemin is released from erythrocytes during malaria infection and is thought to contribute to the inflammatory response during parasitemia [14]. To further elucidate the interaction between malaria and complement, we examined the ability of hemin to induce complement activation in whole blood from healthy controls. Incubation of hemin caused a dose-dependent complement activation detected as generation of sC5b-9 (Figure 3A). Hemin at 1000 μg/ml was significantly increased over the buffer control and was used in the following experiments. Hemin caused a strong complement activation detected as C3bc, C5a, and sC5b-9 formation (Figure 3B-D). Complement inhibitors blocking the cleavage of C3 (compstatin) completely prevented generation of all three activation products whereas blocking of C5 (eculizumab) completely prevented generation of C5a and sC5b-9 (Figure 3). As expected, none of the activation fragments were affected by the C5aR1 inhibitor (Figure 3).

Effect of complement inhibition on hemin-induced cytokine release

We have previously reported that these malaria patients are characterized by increased plasma levels of several cytokines as compared with healthy controls [22], and as described above, plasma levels of IL-6, IL-8/CXCL8, MCP-1/CCL2 and MIP-1β/CCL4 were significantly correlated with plasma levels of sC5b-9 (Figure 2). Interestingly, hemin induced a robust release of IL-6, IL-8/CXCL8 and MCP-1/CCL2, but not MIP-1β/CCL4, in whole blood after culturing for four hours (Figure 4). The induction of IL-6, IL-8/CXCL8 and MCP-1/CCL2

and the complement dependency thereof was then examined by the use of complement inhibitors as described above. The levels of all three were all significantly lower in the presence of all tested complement inhibitors, including C5aR1 inhibition ($p<0.01$) (Figure 4). No significant differences were observed between the respective inhibitors, implying downstream C5aR1 inhibition as effective as upstream C3 inhibition. We have previously showed a particularly strong association of IL-8/CXCL8 and disease severity in these malaria patients and it is noteworthy that the hemin-induced IL-8/CXCL8 response was particularly prominent (13-fold increase over basal) and this response was substantially counteracted by complement inhibition (Figure 4).

Effect of complement inhibition on hemin-induced CD11b expression

Up-regulation of adhesion molecules on leukocytes like CD11b is of importance for vascular inflammation during malaria infection. Therefore, the ability of hemin to induce CD11 expression on leukoytes in whole blood was tested. Early hemin-induced leukocyte activation was detected as significant ($p<0.001$) up-regulation of CD11b on granulocytes (Figure 5). The increase was substantially inhibited in the presence of complement inhibitors. Hemin also induced CD11b expression on monocytes but the effect of complement inhibitors was in the monocyte population quantitatively marginal.

Hemozoin induces complement activation *ex vivo* in whole blood

As mentioned, the malaria parasite produces crystalline hemozoin upon the breakdown of hemoglobin. To examine whether hemozoin behaved similar to hemin with respect to complement activation and IL-8/CXCL8 release, whole blood was incubated with *P. falciparum*-hemozoin. This caused a significant ($p<0.01$) complement activation detected as generation of sC5b-9, which was abolished by C3 and C5 blockage (Figure 6A). Importantly,

similarly to previous observations with hemin, the IL-8/CXCL8 response was significantly ($p<0.05$) increased upon exposure of hemozoin, and the effect was substantially inhibited by all three complement inhibitors (Figure 6B).

Discussion

We demonstrate here that patients with *P. falciparum* malaria have increased complement activation systemically as compared to healthy controls, as shown by increased levels of sC5b-9, demonstrating that the complement cascade is activated to the very end during falciparum malaria. In particular, high sC5b-9 levels were found in patients with a more severe form of the disease as compared to a milder variant, and correlated with levels of certain inflammatory cytokines (i.e., IL-6, IL-8/CXCL8, MCP-1/CCL2 and MIP-1β/CCL4) that also were elevated in these patients. Furthermore, we document a causal association between complement activation and three of these cytokines in *ex vivo* experiments, where hemin and hemozoin-induced cytokine release, and in particular IL-8/CXCL8, was found to be complement-mediated.

Increased levels of sC5b-9 in malaria patients correlating to disease severity are in line with some previous reports. A small cohort (n=15) of healthy volunteers experimentally infected with the *P. falciparum* had increased levels of sC5b-9 in the early stage of the infection [10]. In another study, 23 patients with severe *falciparum* malaria had increased levels of sC5b-9 together with the complement activation fragment Bb [11]. C5a elevation has been reported in children with cerebral malaria [27] and in primigravid women with placental malaria [28]. Evidence on complement components consumption as a result of malaria paroxysm is also reported in some older studies [29-31].

In the present study we extend these previous findings by examining a relatively large population of adult patients with *P. falciparum* infection, showing a marked increase in sC5b-9 in these patients with particularly high levels in those with the most severe disease, significantly correlated with plasma levels of IL-6, IL-8/CXCL8, MCP-1/CCL2 and MIP-1β/CCL4. It is not surprising to detect products of complement activation together with a

response in pro-inflammatory cytokines in an inflammatory disease as malaria, and we cannot exclusively state that the cytokines elevated here are a result of complement activation. Nevertheless, it is interesting that these particular mediators correlated to sC5b-9. Since complement is a pattern recognition system and early activated during infection, it is tempting to speculate that complement activation is among the factors triggering cytokine release. Induction of IL-8/CXCL8 and MCP-1/CCL2 regularly follows complement activation induced by a number of stimuli and is reduced in complement-deficient blood or in the presence of complement inhibitors [17, 32-37]. Sublytic levels of membrane C5b-9 can also induce direct secretion of the IL-8/CXCL8 and MCP-1/CCL2 chemokines from endothelial cells [38, 39]. This finding could be relevant for the situation in severe *falciparum* malaria.

Our findings in the *ex vivo* part of the study may further support a link between complement activation and induction of certain inflammatory cytokines during *falciparum* malaria. We show that hemin and hemozoin, which are released upon erythrocyte rupture, are both potent complement activators in human whole blood and that this activation subsequently leads to the generation of pro-inflammatory molecules, mediated by the interaction of C5a to the C5aR1 as targeted inhibition of C3 and C5 activation were equally efficient to that of C5aR1 inhibition. Rupture of parasitized erythrocytes exposes the innate immune system to a variety of potential activating agents, which besides hemin and hemozoin include plasmodium DNA and glycosylphosphatidylinositol anchors, which can contribute to the innate immune response [40, 41]. Hemin and hemozoin are released in large quantities [42] have respectively been proposed as modulators of inflammation in malaria [12, 13], and as for hemin, also in hemolytic diseases in general [16, 43]. Here, they were hypothesized them to be potential agents responsible for triggering complement activation during *falciparum* malaria. The form of hemozoin tested was the natural variant produced by the *P. falciparum* parasite in culture. Some divergent effects have been reported after comparing

natural and synthetic hemozoin, which might be attributed to further lipids, DNA and proteins bound to natural hemozoin [12]. Since the aim of the present work was to use the most physiologically relevant form of malaria pigment, natural hemozoin was employed without being delipidized or freed from proteins and DNA.

Hemin, which represents the Fe^{3+}-oxidized form of heme, is liberated extracellular from the hemoglobin released upon erythrocyte rupture and is derived via reactive oxygen species [44]. We found hemin to activate complement in whole blood culture in a dose-dependent manner, and a concentration of 1000 μg/ml was chosen for further experiments. Hemin is efficiently scavenged by hemopexin in plasma [45], and concentrations of 100 μg/ml hardly cause activation above background levels. Hemin has previously been shown to modulate complement activation. Frimat *et al.* demonstrated that hemin was a potent activator of the alternative pathway [46], simultaneously inhibiting classical complement activation, as shown previously [47]. We found both hemozoin and hemin to be able to activate complement, hemozoin already at 10 μg/ml, a concentration at which hemin did not generate any sC5b-9. Nonetheless, both hemin and hemozoin potently induced IL-8/CXCL8 release, the cytokine most strongly associated with disease severity in patients, and this response was markedly abolished by complement inhibition, further supporting a link between complement activation and IL-8/CXCL8 release in *falciparum* malaria.

Hemin-induced granulocyte activation was substantially complement-dependent, whereas the corresponding monocyte activation was not. This is consistent with a previous report showing hemin-induced activation of neutrophils and a subsequent release of IL-8/CXCL8 [16]. Thus, whereas the effect on granulocytes was apparent, it is hard to draw any conclusions on which physiological consequences complement inhibition has on the hemin-induced monocyte activation.

Interaction of C5a with the C5aR1 seems to be the central mechanism for the induction of the cytokines that we found up-regulated *ex vivo*. C5a is a main contributor of complement-mediated inflammatory response, implicated in various clinical frames, *e.g.* sepsis [48], sharing similarities with the inflammatory response in malaria [49, 50]. The impact of C5 in malaria is highlighted in studies of experimental malaria in mice. C5 deficient mice are shown to be resistant to infection with *P. berghei* ANKA as compared to C5 sufficient mice, and they do not develop experimental cerebral malaria [51, 52]. Both C5a interaction with C5aR1 [27, 51] and membrane incorporation of C5b-9 [52] have been described as harmful C5-dependent mechanisms in murine experimental malaria. C5a is also proposed as a mediator for cytokine release in placental malaria [28] and seems to play a key role for the effects observed in the present study. In addition to induction of certain cytokines, the hemin-induced up-regulation of CD11b on granulocytes seemed to be dependent on C5aR1 activation. IL-8/CXCL8 is a potent chemoattractant and activator of granulocytes, and the combined C5aR1-dependent activation of IL-8/CXCL8 and the adhesion molecule CD11b in granulocytes in hemin-exposed whole blood, may suggest an important role for complement in granulocyte activation during *falciparum* malaria.

Conclusions

We postulate complement activation to be an important component in malaria pathophysiology, contributing to triggering a systemic inflammatory response and exacerbation of the disease. We show that hemozoin and hemin are potent complement activators in whole blood and that the resulting inflammatory response is largely dependent on interaction of C5a with the C5aR1. Translation of *ex vivo* findings to the *in vivo* situation should be made with caution. Still, our findings underscore C5a as a potential contributor to the malaria pathogenesis, and the C5a interaction with C5aR1 as a potential target for the deleterious pro-inflammatory response in malaria.

Acknowledgements

The authors are indebted to all the patients and their next of kin participating in this study, to the healthy controls, to the medical doctors, to the nurses and the nurses' aides in the medical wards, to the Intensive Care Unit, to the laboratory coordinators and laboratory personnel in the General Laboratory, the Microbiological Laboratory, and the Anatomic Pathology Laboratory in the Central Hospital of Maputo, Mozambique. Special thanks are extended to Einar Sverre Berg at the Department of Virology, The Norwegian Institute of Public Health, Oslo, Norway, for performing the RNA/DNA-nucleic extraction and HIV- PCR, to Christel Haanshuus at the National Center for Tropical Medicine and Imported Diseases, Bergen, Norway, for performing the malaria PCR and sequencing, and to Judith Krey Ludviksen (Research Laboratory Nordland Hospital, Bodø, Norway) for performing the cytokine analyses.

Authors' contributions

AB: *In vivo* and *ex vivo* study design, data collection, analysis, interpretation, statistical calculation and manuscript writing.

KO: *Ex vivo* study design, analyses, interpretation and manuscript writing

SP: *In vivo* study design, preparation for data collection and manuscript writing.

MG: *In vivo* data collection and manuscript writing.

CD: *In vivo* data collection and manuscript writing.

SN: *Ex vivo* study design, analyses, interpretation and manuscript writing

MN: *Ex vivo* study design, analyses, interpretation and manuscript writing

TU: *In vivo* result interpretation, ex vivo study design, manuscript writing.

MP: Production of hemozoin, manuscript writing.

GG: Production of hemozoin, manuscript writing.

TEM: *In vivo* analysis and interpretation, *ex vivo* study design, analyses and manuscript writing.

PA: *In vivo* and *ex vivo* study design, data collection, analysis, interpretation and manuscript writing.

NL: *In vivo* and *ex vivo* study design, data collection, analysis, interpretation and manuscript writing.

PN: *Ex vivo* study design, analyses, interpretation and manuscript writing.

All authors approved the final version of the manuscript.

Conflicts of interest: We declare that the authors have no conflicts of interest.

References

[1] Murray CJ, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, Haring D, et al. Global malaria mortality between 1980 and 2010: a systematic analysis. Lancet 2012;379:413-31.

[2] White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM. Malaria. Lancet 2014;383:723-35.

[3] Holers VM. Complement and its receptors: new insights into human disease. Annu Rev Immunol 2014;32:433-59.

[4] Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 2010;11:785-97.

[5] Harboe M, Mollnes TE. The alternative complement pathway revisited. J Cell Mol Med 2008;12:1074-84.

[6] Barratt-Due A, Pischke SE, Brekke OL, Thorgersen EB, Nielsen EW, Espevik T, et al. Bride and groom in systemic inflammation--the bells ring for complement and Toll in cooperation. Immunobiology 2012;217:1047-56.

[7] Brandtzaeg P, Mollnes TE, Kierulf P. Complement activation and endotoxin levels in systemic meningococcal disease. J Infect Dis 1989;160:58-65.

[8] Dulaney AD. The complement content of human sera with especial reference to malaria. J Clin Invest 1948;27:320-6.

[9] Schindler R, Voller A. A comparison of complement-fixation and fluorescent antibody methods in a longitudinal study of simian malaria infection. Bull World Health Organ 1967;37:669-74.

[10] Roestenberg M, McCall M, Mollnes TE, van Deuren M, Sprong T, Klasen I, et al. Complement activation in experimental human malaria infection. Trans R Soc Trop Med Hyg 2007;101:643-9.

[11] Wenisch C, Spitzauer S, Florris-Linau K, Rumpold H, Vannaphan S, Parschalk B, et al. Complement activation in severe Plasmodium falciparum malaria. Clin Immunol Immunopathol 1997;85:166-71.

[12] Boura M, Frita R, Gois A, Carvalho T, Hanscheid T. The hemozoin conundrum: is malaria pigment immune-activating, inhibiting, or simply a bystander? Trends Parasitol 2013;29:469-76.

[13] Olivier M, Van Den Ham K, Shio MT, Kassa FA, Fougeray S. Malarial Pigment Hemozoin and the Innate Inflammatory Response. Front Immunol 2014;5:25.

[14] Ferreira A, Balla J, Jeney V, Balla G, Soares MP. A central role for free heme in the pathogenesis of severe malaria: the missing link? J Mol Med (Berl) 2008;86:1097-111.

[15] Jakeman GN, Saul A, Hogarth WL, Collins WE. Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. Parasitology 1999;119 (Pt 2):127-33.

[16] Graca-Souza AV, Arruda MA, de Freitas MS, Barja-Fidalgo C, Oliveira PL. Neutrophil activation by heme: implications for inflammatory processes. Blood 2002;99:4160-5.

[17] Lappegard KT, Christiansen D, Pharo A, Thorgersen EB, Hellerud BC, Lindstad J, et al. Human genetic deficiencies reveal the roles of complement in the inflammatory network: lessons from nature. Proc Natl Acad Sci U S A 2009;106:15861-6.

[18] Mollnes TE, Brekke OL, Fung M, Fure H, Christiansen D, Bergseth G, et al. Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. Blood 2002;100:1869-77.

[19] Berg A, Patel S, Aukrust P, David C, Gonca M, Berg ES, et al. Increased severity and mortality in adults co-infected with malaria and HIV in Maputo, Mozambique: a prospective cross-sectional study. PLoS One 2014;9:e88257.

[20] WHO (2011) Guidelines for the treatment of malaria, second edition. Available: http://whqlibdoc.who.int/publications/2010/9789241547925_eng.pdf Accessed 2014 Jan 12.

[21] Bergseth G, Ludviksen JK, Kirschfink M, Giclas PC, Nilsson B, Mollnes TE. An international serum standard for application in assays to detect human complement activation products. Mol Immunol 2013;56:232-9.

[22] Berg A, Patel S, Gonca M, David C, Otterdal K, Ueland T, et al. Cytokine Network in Adults with Falciparum Malaria and HIV-1: Increased IL-8 and IP-10 Levels Are Associated with Disease Severity. PLoS One 2014;9:e114480.

[23] Polimeni M, Valente E, Ulliers D, Opdenakker G, Van den Steen PE, Giribaldi G, et al. Natural haemozoin induces expression and release of human monocyte tissue inhibitor of metalloproteinase-1. PLoS One 2013;8:e71468.

[24] Deroost K, Tyberghein A, Lays N, Noppen S, Schwarzer E, Vanstreels E, et al. Hemozoin induces lung inflammation and correlates with malaria-associated acute respiratory distress syndrome. Am J Respir Cell Mol Biol 2013;48:589-600.

[25] Qu H, Ricklin D, Bai H, Chen H, Reis ES, Maciejewski M, et al. New analogs of the clinical complement inhibitor compstatin with subnanomolar affinity and enhanced pharmacokinetic properties. Immunobiology 2013;218:496-505.
[26] Finch AM, Wong AK, Paczkowski NJ, Wadi SK, Craik DJ, Fairlie DP, et al. Low-molecular-weight peptidic and cyclic antagonists of the receptor for the complement factor C5a. J Med Chem 1999;42:1965-74.
[27] Kim H, Erdman LK, Lu Z, Serghides L, Zhong K, Dhabangi A, et al. Functional roles for C5a and C5aR but not C5L2 in the pathogenesis of human and experimental cerebral malaria. Infect Immun 2014;82:371-9.
[28] Conroy A, Serghides L, Finney C, Owino SO, Kumar S, Gowda DC, et al. C5a enhances dysregulated inflammatory and angiogenic responses to malaria in vitro: potential implications for placental malaria. PLoS One 2009;4:e4953.
[29] Greenwood BM, Brueton MJ. Complement activation in children with acute malaria. Clin Exp Immunol 1974;18:267-72.
[30] Neva FA, Howard WA, Glew RH, Krotoski WA, Gam AA, Collins WE, et al. Relationship of serum complement levels to events of the malarial paroxysm. J Clin Invest 1974;54:451-60.
[31] Phanuphak P, Hanvanich M, Sakulramrung R, Moollaor P, Sitprija V, Phanthumkosol D. Complement changes in falciparum malaria infection. Clin Exp Immunol 1985;59:571-6.
[32] Bergseth G, Lambris JD, Mollnes TE, Lappegard KT. Artificial surface-induced inflammation relies on complement factor 5: proof from a deficient person. Ann Thorac Surg 2011;91:527-33.
[33] Gueler F, Rong S, Gwinner W, Mengel M, Brocker V, Schon S, et al. Complement 5a receptor inhibition improves renal allograft survival. J Am Soc Nephrol 2008;19:2302-12.
[34] Lappegard KT, Fung M, Bergseth G, Riesenfeld J, Mollnes TE. Artificial surface-induced cytokine synthesis: effect of heparin coating and complement inhibition. Ann Thorac Surg 2004;78:38-44; discussion -5.
[35] Rokstad AM, Brekke OL, Steinkjer B, Ryan L, Kollarikova G, Strand BL, et al. The induction of cytokines by polycation containing microspheres by a complement dependent mechanism. Biomaterials 2013;34:621-30.
[36] Samstad EO, Niyonzima N, Nymo S, Aune MH, Ryan L, Bakke SS, et al. Cholesterol crystals induce complement-dependent inflammasome activation and cytokine release. J Immunol 2014;192:2837-45.
[37] Selvan RS, Kapadia HB, Platt JL. Complement-induced expression of chemokine genes in endothelium: regulation by IL-1-dependent and -independent mechanisms. J Immunol 1998;161:4388-95.
[38] Kilgore KS, Flory CM, Miller BF, Evans VM, Warren JS. The membrane attack complex of complement induces interleukin-8 and monocyte chemoattractant protein-1 secretion from human umbilical vein endothelial cells. Am J Pathol 1996;149:953-61.
[39] Kilgore KS, Schmid E, Shanley TP, Flory CM, Maheswari V, Tramontini NL, et al. Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-kappa B activation. Am J Pathol 1997;150:2019-31.
[40] Boutlis CS, Riley EM, Anstey NM, de Souza JB. Glycosylphosphatidylinositols in malaria pathogenesis and immunity: potential for therapeutic inhibition and vaccination. Curr Top Microbiol Immunol 2005;297:145-85.
[41] Kalantari P, DeOliveira RB, Chan J, Corbett Y, Rathinam V, Stutz A, et al. Dual engagement of the NLRP3 and AIM2 inflammasomes by plasmodium-derived hemozoin and DNA during malaria. Cell Rep 2014;6:196-210.
[42] Omodeo-Sale F, Motti A, Dondorp A, White NJ, Taramelli D. Destabilisation and subsequent lysis of human erythrocytes induced by Plasmodium falciparum haem products. Eur J Haematol 2005;74:324-32.
[43] Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. JAMA 2005;293:1653-62.
[44] Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, Epiphanio S, et al. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. Nat Med 2007;13:703-10.
[45] Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. Toxicol Lett 2005;157:175-88.
[46] Frimat M, Tabarin F, Dimitrov JD, Poitou C, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, et al. Complement activation by heme as a secondary hit for atypical hemolytic uremic syndrome. Blood 2013;122:282-92.
[47] Roumenina LT, Radanova M, Atanasov BP, Popov KT, Kaveri SV, Lacroix-Desmazes S, et al. Heme interacts with c1q and inhibits the classical complement pathway. J Biol Chem 2011;286:16459-69.
[48] Ward PA. The harmful role of c5a on innate immunity in sepsis. J Innate Immun 2010;2:439-45.
[49] Clark IA, Alleva LM, Budd AC, Cowden WB. Understanding the role of inflammatory cytokines in malaria and related diseases. Travel Med Infect Dis 2008;6:67-81.

[50] Clark IA, Budd AC, Alleva LM, Cowden WB. Human malarial disease: a consequence of inflammatory cytokine release. Malar J 2006;5:85.

[51] Patel SN, Berghout J, Lovegrove FE, Ayi K, Conroy A, Serghides L, et al. C5 deficiency and C5a or C5aR blockade protects against cerebral malaria. J Exp Med 2008;205:1133-43.

[52] Ramos TN, Darley MM, Hu X, Billker O, Rayner JC, Ahras M, et al. Cutting edge: the membrane attack complex of complement is required for the development of murine experimental cerebral malaria. J Immunol 2011;186:6657-60.

Figures

Figure 1. Complement activation as measured by sC5b-9 and relation to malaria severity score. Plasma samples from patients with *P. falciparum* malaria were analyzed for the complement activation product sC5b-9. Patients were divided into two groups based on Malaria Severity Score (MSS) <3 (n=114) or ≥3 (n=15), and related to healthy controls (HC) (n=52). Levels of sC5b-9 are presented in the figure as box-and-whiskers plot with 5-95 percentile range, *=$p<0.05$, ***$p<0.001$.

Figure 2: Correlation of sC5b-9 to IL-6, IL-8/CXCL8, MCP-1/CCL2 and MIP-1β/CCL4 in the malaria patients. Correlation of sC5b-9 to cytokines in plasma samples of malaria patients (n=131) was tested using Spearman's Rank Order Correlation on logarithmic values. Significant ($p<0.01$) positive correlation was found between sC5b-9 and each of the following variables, with $r_s=0.63$ for IL-6 (A), $r_s=0.52$ for IL-8/CXCL8 (B), $r_s=0.62$ for MCP-1/CCL2 (C) and $r_s=0.62$ for MIP-1β/CCL4 (D) respectively. Linear values are plotted in graphs on logarithmic scale.

Figure 3. Complement activation by hemin in whole blood *ex vivo*.

Hemin was incubated for four hours in whole blood. Dose-dependent complement response to hemin was tested with hemin 10-1000 μg/mL; complement activation was detected as sC5b-9 (A). Hemin (1000 μg) was then incubated in the presence or absence of complement inhibitors at the level of C3 (compstatin), C5 (eculizumab), and C5aR1 (C5aRag). Complement activation was detected as generation of C3bc (B), C5a (C) and sC5b-9 (D). Zymosan (1000 μg/ml) served as positive control. Data are presented as means and 95 % confidence intervals; n=6; *$p<0.05$, ***$p<0.001$, ns=non-significant.

Dose-dependent complement response to hemin was investigated by incubating hemin 10-1000 μg/mL in whole blood for four hours.

Figure 4. Cytokine release *ex vivo* in whole blood incubated with hemin with or without complement inhibitors.

Hemin-induced increase in IL-6 (A), IL-8/CXCL8 (B) and MCP-1/CCL2 (C) in comparison to the buffer control is depicted. Complement inhibition at all tested levels (C3, C5, C5aR1) are shown. Data are presented as means and 95 % confidence intervals; n=6; *p<0.05, **p<0.01, ***p<0.001.

Figure 5. Effect of hemin on up-regulation of CD11b on granulocytes in whole blood.

Up-regulation of CD11b on granulocytes and monocytes was examined after 15 minutes whole blood incubation of hemin with or without complement inhibitors. Expression of CD11b specifically analyzed in the granulocyte population measured as Mean Fluorescence Intensity (MFI) is shown in the figure. Complement inhibitors are as described in Figure 3. Data are presented as means and 95 % confidence intervals; n=6; **p<0.01, ***p<0.001.

Figure 6. Effect of hemozoin on complement activation *ex vivo* in a whole blood model.

Hemozoin was incubated for four hours in the presence or absence of complement inhibitors at the level of C3 (compstatin), C5 (eculizumab), and C5aR1 (C5aRA). The degree of complement activation was investigated as sC5b-9 (A) and the effect of complement inhibitors on the IL-8/CXCL8 release was investigated (B). Data are presented as means and 95 % confidence intervals; n=5 (C5aRA, n=4; LPS, n=3); *p<0.05, **p<0.01, ns=non-significant.

Fig 1

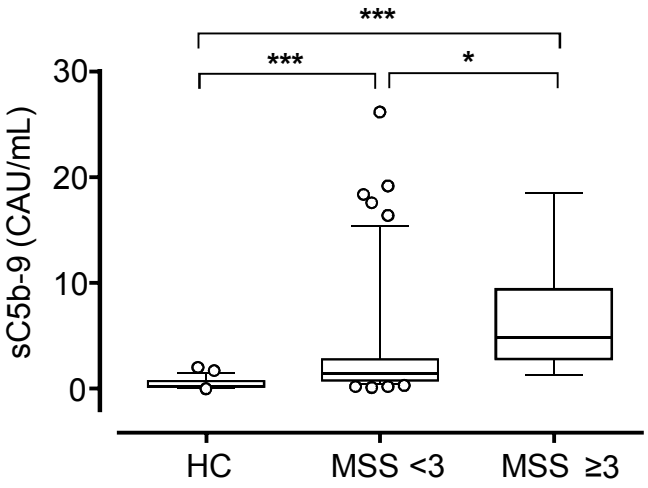


Fig 2

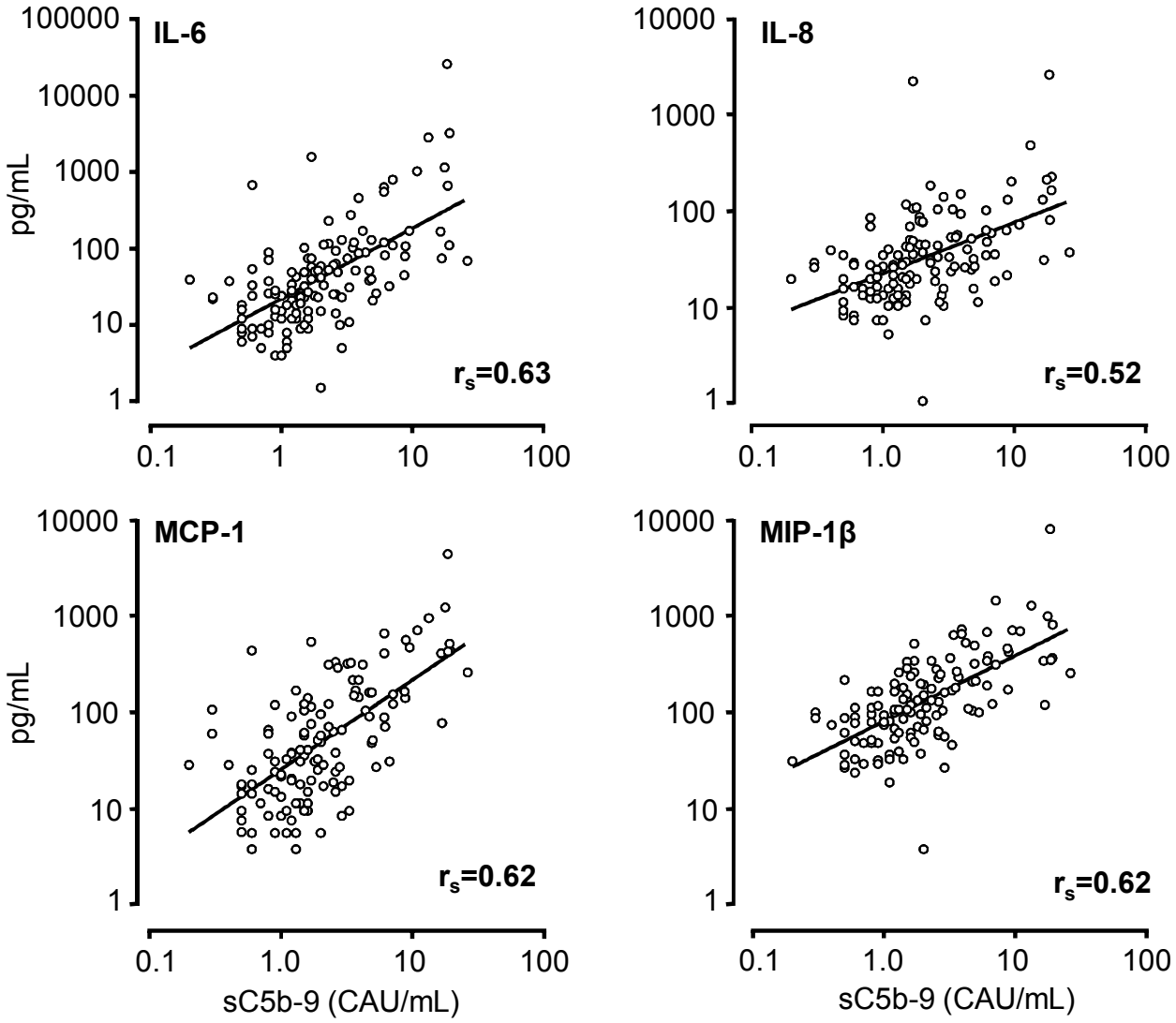

IL-6
pg/mL
100000
10000
1000
100
10
1
0.1
1
10
100
r_s=0.63
IL-8
10000
1000
100
10
1
0.1
1.0
10
100
r_s=0.52
MCP-1
pg/mL
10000
1000
100
10
1
0.1
1.0
10
100
sC5b-9 (CAU/mL)
r_s=0.62
MIP-1β
10000
1000
100
10
1
0.1
1.0
10
100
sC5b-9 (CAU/mL)
r_s=0.62

Fig 3

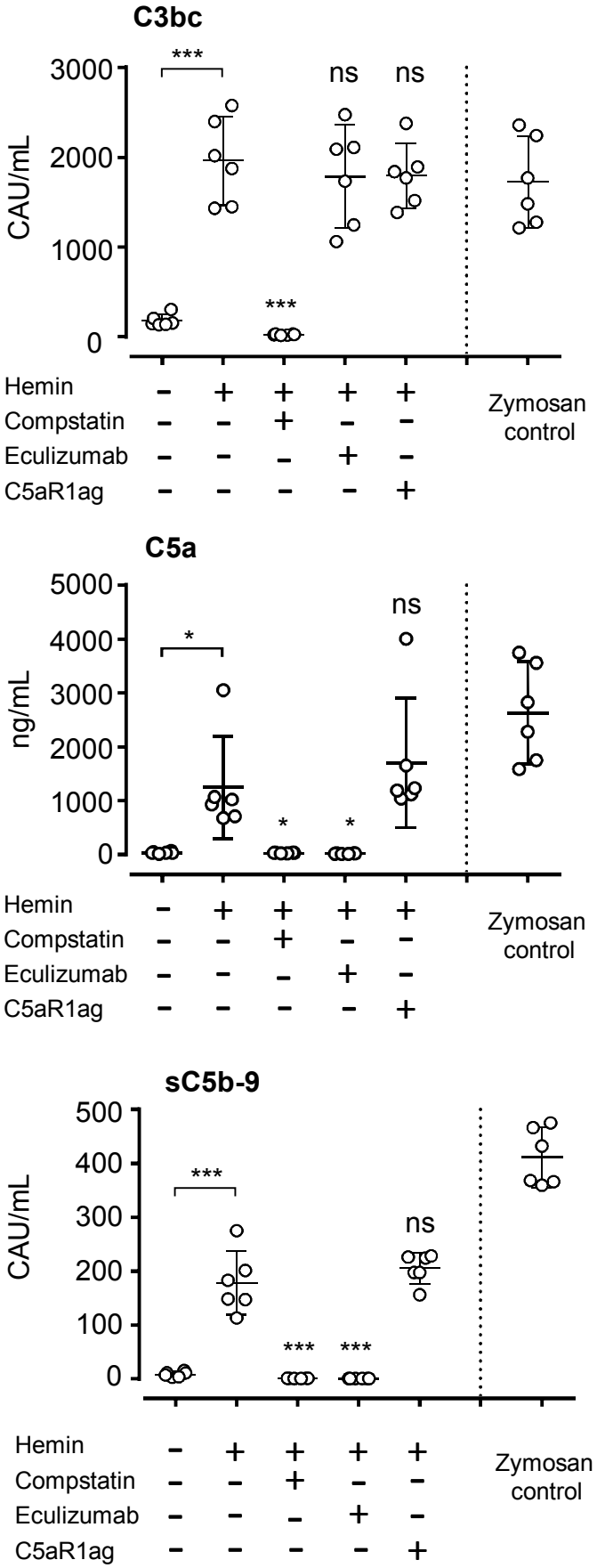

C3bc
CAU/mL
3000
2000
1000
0
***
ns
ns
***
Hemin
Compstatin
Eculizumab
C5aR1ag
Zymosan control
C5a
ng/mL
5000
4000
3000
2000
1000
0
*
ns
*
*
Hemin
Compstatin
Eculizumab
C5aR1ag
Zymosan control
sC5b-9
CAU/mL
500
400
300
200
100
0
***
ns
***
***
Hemin
Compstatin
Eculizumab
C5aR1ag
Zymosan control

Fig 4

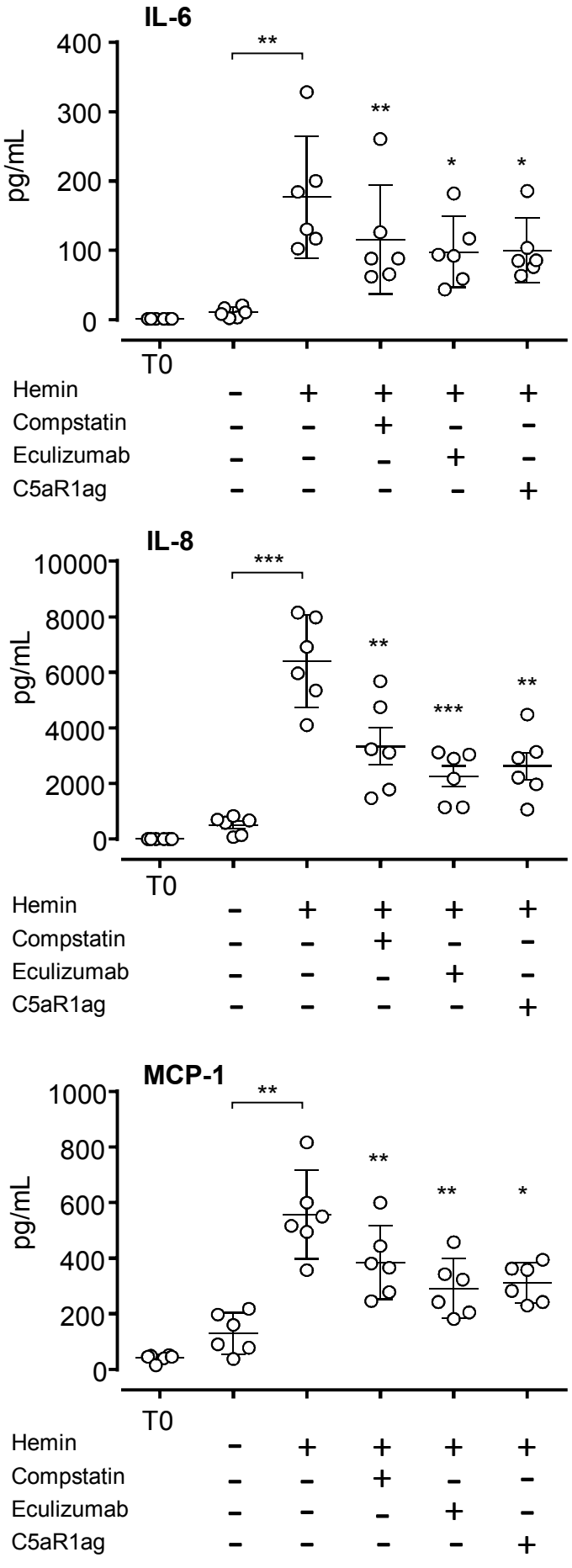

IL-6
400
300
200
100
0
pg/mL
**
**
*
*
T0
Hemin - + + + +
Compstatin - - + - -
Eculizumab - - - + -
C5aR1ag - - - - +
IL-8
10000
8000
6000
4000
2000
0
pg/mL
***
**
***
**
T0
Hemin - + + + +
Compstatin - - + - -
Eculizumab - - - + -
C5aR1ag - - - - +
MCP-1
1000
800
600
400
200
0
pg/mL
**
**
**
*
T0
Hemin - + + + +
Compstatin - - + - -
Eculizumab - - - + -
C5aR1ag - - - - +

Fig 5

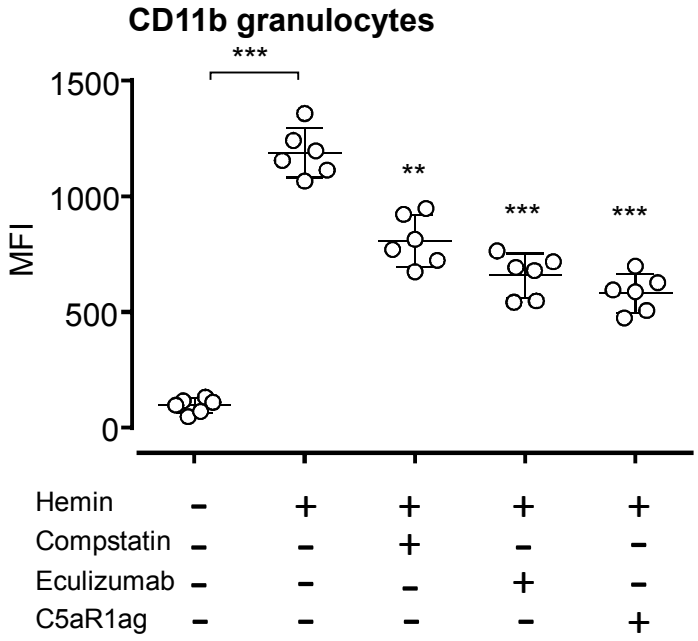
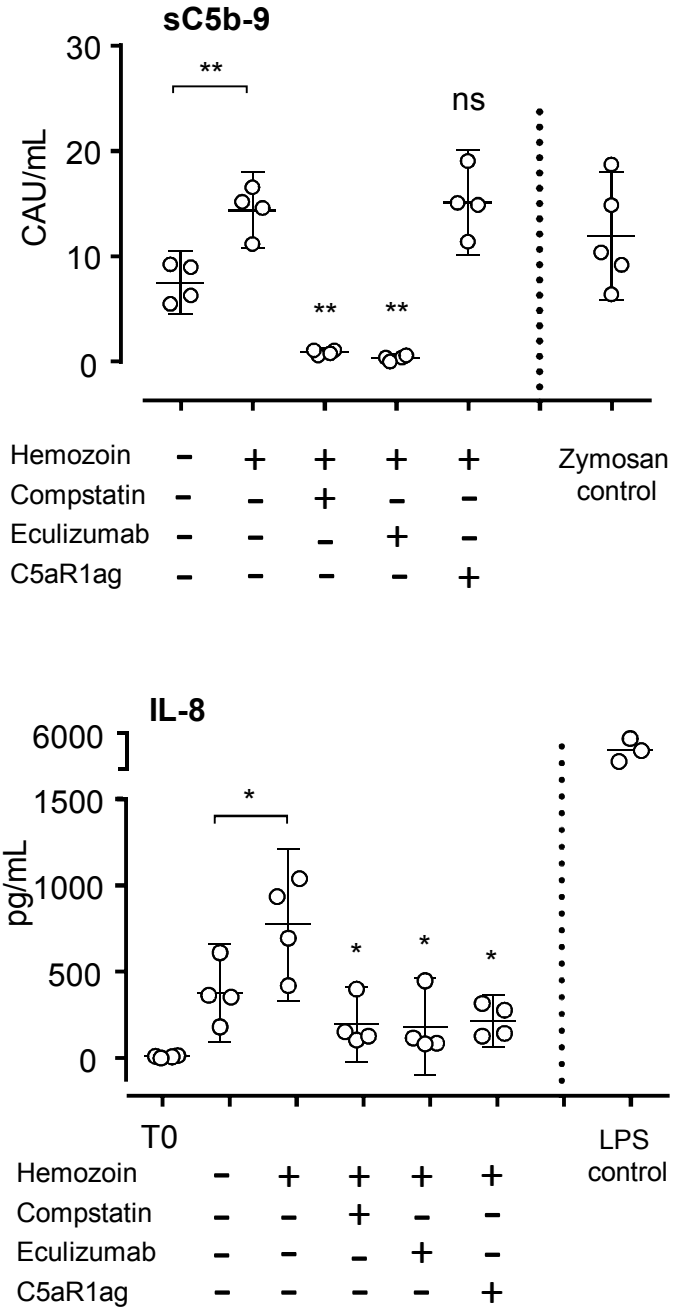

CD11b granulocytes
MFI
1500
1000
500
0
***
**
***
***
Hemin - + + + +
Compstatin - - + - -
Eculizumab - - - + -
C5aR1ag - - - - +

Fig 6


sC5b-9
CAU/mL
30
20
10
0
**
ns
**
**
Hemozoin - + + + +
Compstatin - - + - -
Eculizumab - - - + -
C5aR1ag - - - - +
Zymosan control
IL-8
pg/mL
6000
1500
1000
500
0
*
*
*
*
T0
LPS control
Hemozoin - + + + +
Compstatin - - + - -
Eculizumab - - - + -
C5aR1ag - - - - +